Sickle Cell Anemia Research and a Recombinant DNA Technique

Letters from:

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The development of any new technology that could increase the frequency of homologous recombination by three to six orders of magnitude over that seen in normal mammalian cells would represent a major breakthrough. The report by Cole-Strauss *et al.* (6 Sept. 1996, p. 1386), in which an RNA-DNA hybrid oligonucleotide carrying the wild-type β^A sequence was used to correct 50 to 80% of B cells that carry a mutant β^S-globin locus, therefore gained much attention from the scientific community.

The possibility that this unexpected result could be a consequence of a potential artifact was previously raised in a letter by Thomas and Cappechi (7 Mar., p. 1404). Careful examination of the data presented in figure 4 (p. 1388) of the report by Cole-Strauss suggests that such an explanation is likely. As noted by Cole-Strauss *et al.*, wild-type cells are polymorphic at the third base of codon 2, with a mixture of cytosine (C) and thymidine (T) at this position. The sequence analysis of β^A (first row) shows this clearly. The mutant β^S gene does not have this polymorphism, and only C is present at this position (third row). Because the "correcting" oligonucleotides SC1 and SC2 also contain C at this position, recombination should not introduce any changes at this site of the β^S allele. After incubation with SC1 (fourth row), however, where the dramatic correction of nearly 50% A in the mutants that previously only had T at base 2 of codon 6 is in evidence, one can also see that there may be 25% T at base 3 of codon 2. There should be 100% C. Such a result might be expected to occur if one had a population containing equal quantities of the mutant and wild-type cells, because there does not appear to be any simpler explanation for the change at the third base of codon 2. Thus, this rate of recombination appears to be a result of contamination of mutant cells by wild-type cells, and the perceived frequency of recombination is equal to the percentage of contamination.

Consistent with this explanation, experiments with the SC2 oligonucleotide (figure 4A, row 5 of the report) also show that the proportion of C is greatly increased at the third base in codon 2. Because the

mutant cells contain only C at this position, while the wild-type cells are polymorphic, we think it is likely that, in this case, contamination of the wild-type cells by mutant cells led to the observed high frequency conversion of the β^A to the β^S allele at base 2 of codon 6. We think that this alternative argument of significant contamination explains the unexpected effects seen at the third position of codon 2, away from the sickle-cell mutation of interest at codon 6.

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Response: Our work has been aimed at demonstrating the feasibility of using small synthetic oligonucleotides to effect an alteration in a selected gene in vivo. Recognizing that our results represented an improvement in the ability to cause desired changes in genes without the need for selection of the modified cells, we have taken a cautious approach in our presentation, in performing controls, replicating each experiment, and avoiding statements of specific conversion frequency. This work represented a logical extension of our previous publication (1), in which we demonstrated highly efficient targeting of an episomal target in Chinese hamster ovary (CHO) cells with oligonucleotides of similar design. In all of our efforts, we have considered three types of potential artifacts.

1) PCR. There is a potential for polymerase chain reaction (PCR)-mediated artifacts and mutations when an analysis is based solely on the results of PCR amplification. We based our detection and quantitation in (1) on an assay that did not require PCR (we used oligonucleotide hybridization of unamplified episomal DNA). In later work (2), the assays included a Southern blot (DNA) analysis, where no DNA amplification was performed. Subsequently, we found that the presence of a vast excess of the mutagenic oligonucleotides in the PCR reaction cannot introduce the desired mutation (3).

2) Contamination. Our awareness of the limits of the conventional homologous recombination methodology (frequency of conversion ranging from 1 to 1000 to 1 in 10^6) led us to select methods of detection effective over a range of frequencies. Apparent conversion can always be argued as contamination or random reversion of a mutation, so we used multiple methods of detection. The automated sequence analysis methodology that uses fluorescent dyes is approximately equal in sensitivity to a properly controlled restriction polymorphism analysis. In response to this criticism, we tested whether a mixture of the β^S and β^A cells would generate the expected results and pattern. We established that when a mixed sequence is detectable by sequencing, it is also evident in the polymorphism assay. Consequently, the lack of Bsu 36 1 digestion (2) in the sickle homozygous cells indicates that the cells

are not, nor did they become, polymorphic. The conversion experiment has been carried out many times, with parallel detection by polymorphism and sequence assays, and although there is variability in the frequency of conversion, we can detect the conversion event in untreated cells by both assays, which indicates that the methodology does represent an advance over existing technologies. The appearance of double and even triple peaks in automated sequence chromatograms is a common occurrence and is rarely indicative of contamination. We have sequenced both strands in several samples to rule out this possibility.

3) Cloning. It is imperative that the stable inheritance of the introduced mutation needs to be demonstrated to rule out trivial explanations. Because we encountered difficulty in cloning the cells used in this study, we have relied on demonstrating conversion of the beta allele in two different cell types, human HeLA and HUH7, using the SC2 oligonucleotides described in the report (2). Both cells are amenable to cloning by limiting dilution, and we have completed experiments that confirm stable genetic changes at the specific base of the β-globin gene. Other workers have recently, independently completed a study showing the targeted conversion of both the alkaline phosphatase and the β globin genes in the genome of HUH7 cells (4).

We are aware that our two papers (1, 2) have led many (including ourselves) to test the method on multiple targets and in different cells. We mentioned in our report (4) the potential variability of different cell types and frequency of conversion by highlighting our lack of success in the TK6 cell line. Future studies should reveal how this technology is applicable to different cell types and genes.

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